Development of Chitosan–SLN Microparticles for chemotherapy: In vitro approach through efflux-transporter modulation

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A B S T R A C T

Drug efflux-transporters serve as a major barrier to anticancer drugs at the target site. One strategy to enhance the therapeutic efficacy of drugs against cancer is to increase their available concentrations at the target site by suppressing or modulating efflux-transporters. This manuscript deals with the development and evaluation of the particle type drug delivery system made of stearic acid (Solid Lipid Nanoparticle – SLN) and chitosan for the delivery of Phenethyl Isothiocyanate (PEITC), a tumor-suppressive agent, through the pulmonary route. The rationale behind the particle type drug delivery system involves a prior release of the efflux-transporter substrate, PEITC, followed by release of the efflux activity of ABC transporters. The efficacy of Chitosan–SLN Microparticles (CSM) as a carrier for PEITC was evaluated by investigating the release profiles of PEITC loaded in CSM and its cytotoxicity in the presence or absence of the efflux-transporter inhibitors. An initial burst release of the inhibitors, followed by gradual, sustained release of PEITC and subsequent increase in cytotoxicity was observed. This finding indicated that the efflux transporter inhibitors significantly affected the PEITC uptake rate by Calu-3 cells. Judging from these results, CSM can be an efficient drug delivery system for the substrates susceptible to the efflux-transporters.

1. Introduction

Efflux-transporters like P-gp (P glycoprotein), MRP (Multi-drug Resistant Protein) and BCRP (Breast cancer Resistant Protein) serve as a major barrier to anticancer drugs. Due to the presence of drug efflux-transporters, pharmacological response from non-penetrative routes of chemotherapy is at most moderate. Randomized trials have demonstrated that the modulation of ABC proteins by cyclosporine A or verapamil HCI increased the therapeutic responses to chemotherapy [1–4]. Therefore, the combination of chemotherapeutic drugs along with drug efflux-transporter inhibitors may provide beneficial effects on chemotherapy.

Isothiocyanates are a dietary constituent of cruciferous vegetables which have anti-tumor activities. More than 25 synthetic and natural isothiocyanates have been identified and tested for their activities. Phenethyl Isothiocyanate (PEITC) is a naturally occurring isothiocyanate which has entered phase 1 clinical trials (National Institute of Cancer) as a preventive agent against lung cancer. PEITC acts through termination of phase 1 stage of ontogenesis and stimulates the enzymatic activity involved in detoxification of carcinogens. PEITC, similar to most cytotoxic drugs, is insoluble in water and a substrate to the efflux proteins, which requires high doses of PEITC to be effective as a chemotherapeutic agent [5]. These limitations have posed the major challenges to the development of an efficient delivery system for PEITC and achievement of its therapeutic concentrations at the tumor cells.

Solid Lipid Nanoparticles (SLN) are in the form of solid lipids (i.e. lipids that are solid at room temperature) and provide an alternative option for encapsulating lipophilic compounds. Protein stabilization could be achieved by a suitable particle formulation or a polymer mixture [6]. Core shell type delivery systems have also been utilized in preparing pH and thermal-responsive particulate systems [7]. SLN were dispersed in chitosan, which is biocompatible and biodegradable hydrophilic polymer and widely used as a drug carrier. Chitosan–SLN Microparticles (CSM) is intended to achieve the greater in vivo efficacy of the loaded drugs by addressing both physiological and formulation drawbacks of the conventional formulations [8,9].

This study is aimed to develop CSM which is made of chitosan and loaded with stearic acid based SLN. The composite CSM system is in the form of microparticles and can be used for the treatment of lung cancer via pulmonary route. It is hypothesized that CSM incorporated with one of the fast releasing efflux inhibitors, such as tamoxifen, verapamil or nifedipine, in the shell and a slower releasing anti-cancer agent, PEITC, in the core would overcome efflux-transporter imposed restrictions on chemotherapy. The encapsulation entity, release profiles and chemotherapeutic efficacy of PEITC in the presence of the efflux transporter inhibitors were evaluated for the assessment of...
in vitro outcomes. Cytotoxic expression of Calu-3 cells exerted by accumulative PEITC was assessed as an index of pharmacological activity of CSM.

2. Materials

Chitosan (Brookfield viscosity 20 cps), Stearic acid, Tween 80, Sodium Taurocholate, light mineral oil, Sulforhodamine 101 acid hydrochloride, (4′,6-Diamidino-2-phenylindole, dilactate (DAPI), Phenylthethylisothiocyanate (PEITC), tamoxifen, verapamil, nifedipine and hexane were obtained from Sigma-Aldrich Co. (St Louis, MO). All materials were of analytical grade. PEITC and nifedipine were handled under low light and stored in amber colored containers due to photosensitivity.

3. Methods

3.1. Preparation of SLN

The formulation was prepared in 2 steps as shown in Fig. 1. In the first stage, SLN was prepared with Stearic acid and Tween 80. In the second stage, the SLN solution is dispersed in the chitosan solution to form Chitosan–SLN Microparticles (CSM).

Stearic acid was mixed thoroughly with PEITC at 70 °C. Tween 80 and sodium taurocholate were added to the mixture and left for melt at 70 °C. To the melted solution, 1 ml of triple distilled water was added at 70 °C. An optically transparent micro-emulsion was obtained by stirring a mixture at 3000 rpm for 10 min. CSM were cross-linked with 0.1% glutaraldehyde to maintain long-term stability.

3.2. Preparation of Chitosan–SLN Microparticles

Chitosan (Brookfield viscosity 20 cps) was dispersed in 1% acetic acid with or without inhibitors. To this solution, 50 mg of SLN were added and a mixture was stirred till a uniform solution was obtained. The chitosan–SLN dispersion was dropped into span 80 (2%) in light mineral oil and stirred at 3000 rpm for 10 min (Fig. 1). CSM were cross linked with 0.1% glutaraldehyde to maintain long-term stability.

3.3. Evaluation of the particle size

The particle size distribution of the freshly prepared Chitosan–SLN Microparticles (CSM) and SLN loaded with PEITC were analyzed using Brookhaven 90 Plus (Brookhaven Instruments Limited, Holtsville, NY) and sodium taurocholate were added to the mixture and left for melt. SLNs were freeze-dried until further usage.

3.4. Scanning electron microscopy study

SLN and CSM were washed with hexane and subsequently lyophilized. The freeze-dried particles were placed on an aluminum sample mount and sputter-coated with gold-palladium to minimize surface charge. The sputter coated samples were then examined for surface morphology using Field-Emission Environmental SEM (Philips XL30, Philips Inc., Hillsboro, OR).

3.5. Confocal microscopy study

CSM were prepared as described above and encapsulated with fluorescent dyes DAPI and sulforhodamine. SLN were labeled with DAPI, whereas chitosan was labeled with sulforhodamine 101. The labeled CSM were washed, freeze-dried and re-dispersed on a glass slide. They were enclosed with a cover-slip. CSM were examined for surface charge. The sputter coated samples were then examined for surface morphology using Field-Emission Environmental SEM (Philips XL30, Philips Inc., Hillsboro, OR).

3.6. Drug loading process

Samples from at least three batches were tested and encapsulation efficiency (EE) was determined using the equation:

\[ EE = \frac{D_o}{D_i} \times 100 \]

D_o is the loaded amount of PEITC and D_i is the initial added amount of PEITC.

The amounts of PEITC in SLN were examined by dissolving them in absolute ethanol solution and subsequently determined by HPLC at 270 nm. The amounts of PEITC were determined by dissolving them in 0.1 N HCl followed by HPLC assay at 270 nm.

3.7. In vitro drug release study

Particle dispersion of PEITC (20 mg) in 5 ml of PBS at pH 7.4 was loaded in the dialysis membrane (3500 kDa) and suspended in 15 ml of modified PBS. The sample (0.1 ml each) was withdrawn at a predetermined time interval and 0.1 ml PBS with Tween 80 (0.01%) was added to maintain sink conditions [11]. The loading doses of three efflux-transporter inhibitors (chitosan to inhibitor load ratio of 10:1) were normalized based on nanoparticle weight and expressed as the percentage of the normalized dose in the release study. The sample was subjected to HPLC assay (Varian 9012 pump; Varian, Paulo Alto, CA) equipped with a UV detector (Waters 484, Waters Co., Milford, MA) and a reversed phase C18 column (5 µm, Phenomenex Inc, Torrance, CA). Samples were analyzed using the isocratic method with a mobile phase containing 30:70 methanol and water [12,13] at 270 nm wavelength. All experiments were performed in triplicate and taken from at least two different batches unless specified otherwise.
medium. PEITC concentration released from the CSM formulations was approximately 20 or 50 μM in the culture medium. CSM which had no encapsulated inhibitor was used as the control. Cells were incubated for up to 6 days and cell growth in each well was determined by Cell Aqueous One solution [17]. The absorbance values at 490 nm represent the viable cell numbers of each well in the plate. The number of days required to inhibit 50% of cell growth (IC50) was determined by analyzing cell survival curves using Graph pad Prism® 4 (Graph pad software Inc., San Diego, CA).

3.9. Statistical analysis

The differences in cell expression and changes in release rates under various experimental conditions were statistically analyzed by a one-way analysis of variance (ANOVA) with pair wise multiple comparisons using the Student–Newman–Keels method. P values ≤0.05 will be considered to be statistically significant.

4. Results

4.1. P glycoprotein expression in Calu-3 cells

The effects of verapamil on the cellular uptake of isothiocyanates were previously reported [18,19]. Using the similar approach, MRP expression in Calu-3 cells was confirmed by PCR experiment. The corresponding mRNA expressions were determined in the experimental cell line (i.e., Calu-3) as well as the positive control cells (i.e., MCF 7 cells).

4.2. Formulation of Chitosan–SLN Microparticles

Stearic acid, chitosan and the components of CSM were selected based on their properties in biocompatibility, solubility, ease of preparation and reproducibility. Stearic acid, Tween 80 and sodium taurocholate, which are lipophilic compounds, form the hydrophilic matrix with chitosan. The status of CSM incorporated with SLN is verified by confocal microscopy. DAPI and sulforhodamine (i.e., fluorescent dyes) were dispersed in stearic acid and chitosan, respectively, and the distribution profiles of SLN in Chitosan–SLN

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Composition and entrapment efficiency of Solid Lipid Nanoparticles (SLN 1, 2, 3) and chitosan (1, 2, 3)</th>
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</thead>
<tbody>
<tr>
<td>Formulation</td>
<td>Composition</td>
</tr>
<tr>
<td>SLN-1</td>
<td>Stearic acid:Tween 80:Sodium taurocholate 2:3:1.5</td>
</tr>
<tr>
<td>SLN-2</td>
<td>2:4:2</td>
</tr>
<tr>
<td>SLN-3</td>
<td>2:5:2.5</td>
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<tr>
<td>CSM-1</td>
<td>–</td>
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All three SLN formulations have different surfactant content, whereas Stearic acid and PEITC content were kept constant. SLN-2 was chosen for further studies based on the best particle % entrapment efficiency. All three CSM formulations have different chitosan content whereas SLN and glutaraldehyde content were kept constant. Chitosan-3 was chosen for further studies based on the best % entrapment efficiency. SLN particle size was determined by Brookhaven 90 plus with at least 400 kcps. CSM particle size was determined by measuring at least 200 particles from 3 different batches.

3.8. Cytotoxicity study

The cytotoxicity of PEITC on Calu-3 cells was determined by assessing the reduction of MTS dye of viable cells upon exposure to various amount of PEITC using CellTiter 96 Aqueous One solution [14,15]. Calu-3 cells were trypsinized and seeded in 96-well plates at a density of 2000 cells per well. After 24 h attachment, the fresh medium containing a serial dilution of CSM was added to the plates. The culture plates were incubated with the culture medium. PEITC concentration released from the CSM formulations was approximately 20 or 50 μM in the culture medium. CSM which had no encapsulated inhibitor was used as the control. Cells were incubated for up to 6 days and cell growth in each well was determined by Cell Aqueous One solution [17]. The absorbance values at 490 nm represent the viable cell numbers of each well in the plate. The number of days required to inhibit 50% of cell growth (IC50) was determined by analyzing cell survival curves using Graph pad Prism® 4 (Graph pad software Inc., San Diego, CA).

To determine the role of the efflux transporter in drug induced cell viability, Calu-3 cells were incubated with four different CSM formulations which contained each of 4 inhibitors. The formulations had three loading amounts of inhibitors resulting in low (~0.1 mM), medium (~1 mM) and high (~10 mM) concentrations in the culture...
Microparticle were identified using confocal microscopy. As shown in Fig. 2, SLN was uniformly distributed in CSM along the internal planes in the particle and no heterogeneous distribution or uneven localization was observed at the particle surface, indicating that SLN was compatibly and homogeneously loaded in CSM.

4.3. Characterization of CSM

The entrapment efficacy of PEITC assessed by dissolving SLN in absolute ethanol ranged from 78 to 86.3%. The high entrapment efficiency of hydrophobic drugs like PEITC is due to its greater partition coefficient, resulting in a longer retention time in the lipid phase. Formulations containing the lowest ratio of surfactant to stearic acid (2:3:1.5) showed the lowest entrapment efficiency with non-uniform particle sizes as shown in Table 1. The lower entrapment efficiency with the highest surfactant ratio (2:5:2.5) than that of (2:4:2) can be attributable to the excess amount of surfactant which increases the solubility of PEITC in water. The relatively higher surfactant ratios (more than 2:5:2.5) produced a turbid solution which did not form the micro-emulsion with stearic acid.

The differences in the entrapment efficiency and particle sizes were within the acceptable range (within 5% standard error between batches). Considering the maximal entrapment efficiency and optimal particle sizes, SLN formulation with a ratio of 2:4:2 (Stearic acid:Tween 80:Sodium Taurocholate) was chosen for further studies. The mean particle size measured by the particle size analyzer was 128 nm ±3.5 with a narrow size distribution ranging from 3 to 10 µm. The SEM micrograph of lyophilized SLN revealed its smooth surface and homogenous shape.

The entrapment efficiency of the SLN in chitosan ranged from 51 to 76%. As the chitosan content increased, the entrapment rate of SLN significantly increased (Table 1). The lower chitosan content (0.8 to 1%) did not yield uniform microparticles and the entrapment efficiency was relatively low (entrapment efficiency <10% and particle size standard error of mean >10%). Although 1.2% chitosan yielded microparticles with relatively lower sizes, the entrapment efficiency of PEITC was still low (32%). Therefore, 2% chitosan at which higher entrapment efficiency of SLN and a uniform particle size were obtained was chosen as an optimal concentration.

4.4. The release profiles of PEITC from SLN and CSM

As shown in Fig. 3, the release rate of PEITC from SLN was faster at the initial stage, slower as time progressed and remained constant till 80% of the loading doses were released. The release profile of PEITC from SLN follows the zero order kinetics which fits the equation of

\[ Y = 105.79X + 2.9935. \]

The release rate of PEITC from CSM was slower than that from SLN. After 24 h, the released amount of PEITC was about 10% of the loading dose. The extrapolated estimation of lag time from the CSM release profile was approximately 1.45 days. The release data were initially assessed by the classical drug release models including the first order, Higuchi and Krosmeyer equations. These conventional equations were poorly correlated with the release profiles of PEITC and failed to fully explain the release data.

The drug release rates were further assessed by modified sigmoid equations [20] to accurately account for the release profile of PEITC using Micromath Scientist software.

\[ F = B/[1 + \exp(-K_a(T-T_{50}))] \]  

(1)

\[ F = A + B/[1 + \exp(-K_a(T-T_{50}))] \]  

(2)

where \( F \) is the fraction of drug released, \( A \) is the percentage of total drug released during phase I (initial burst phase), \( B \) is the percentage of total drug released during phase II, \( K_a \) is the rate constant of drug release during phase II and \( T_{50} \) is the time taken to release 50% of the total drug.

A simple sigmoid model, denoted by Eq. (1), assumes that the release profile is devoid of an initial burst release phase, whereas the modified sigmoid model (Eq. (2)) includes it in term \( A \). When the
involved parameters were examined by Eq. (2), there was a poor
correlation between them. The correlation coefficient of the PEITC
release rate was 0.87 and the value of parameter \( A \) (-4.2\%) was
negative, suggesting that the CSM may have no initial burst release
phase. On the contrary, these data are well corroborated by Eq. ((1)
with the correlation coefficient of 0.98.

A lack of the initial burst release phase of PEITC may be due to its
binding property to the stearic acid matrix. PEITC has similar
lipophilic characteristics to stearic acid rather than hydrated
chitosan, which partially explains the slower release profiles of
PEITC. The initial lag phase of a day can be attributable to the
duration of chitosan matrix to be hydrated before initiation of PEITC
release. A slower release pattern during the initial lag phase might
be due to the lower diffusion rate of PEITC from SLN into the
chitosan matrix. The exponential release profile in phase II seems to
be initiated by PEITC diffusion from chitosan matrix into the
external medium. CSM had a \( T_{50} \) value of 3.4 days, after which the
release rate of PEITC was faster, indicating that there were
disintegration or erosion phases of chitosan matrix. In the eroded
or degraded matrix, the release amount of PEITC arise from the
diffusion process seems negligible.

4.5. The release rates of tamoxifen, nifedipine and verapamil from CSM

SLN containing PEITC along with inhibitors (~0.1 mM, ~1 mM and
~10 mM) of the efflux-transporters including nifedipine, verapamil
HCl or tamoxifen were loaded in CSM. The release profiles of
nifedipine and tamoxifen were similar to each other and had an
initial burst release of approximately 21\% in 6 h as shown in Fig. 4. The
release of the whole loading dose was completed in 5 days. The release
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Fig. 5. Cytotoxicity assay of PEITC in Calu-3 cells. Calu-3 cells were incubated with serial
dilutions of PEITC alone and together with 10 \( \mu M \) of verapamil HCl, nifedipine or
tamoxifen. Experiments were conducted in triplicates at least and the \( IC_{50} \) value was
calculated using Graphpad Prism. The equation fit was at least 0.95. \( N=4 \).

Fig. 6. A) Cytotoxic assay of PEITC in Chitosan–SLN Microparticle with increasing
loading doses of nifedipine. B) Cytotoxic assay of PEITC in Chitosan–SLN Microparticle
with increasing loading doses of Verapamil HCl. C) Cytotoxic assay of PEITC in Chitosan–
SLN Microparticle with increasing loading doses of tamoxifen. The cytotoxic effects of
PEITC on Calu-3 cells were determined as a function of inhibitor concentration.
Cytotoxicity tests were performed in quadruplicate at least.
process of chitosan contributes to overall drug release profiles. The release rate of verapamil HCl was found to be significantly faster than those of tamoxifen and nifedipine (p < 0.05). The faster release rate of verapamil can be attributable to its aqueous solubility, which is much higher (<30 mg/ml) than those of nifedipine and tamoxifen.

4.6. Cytotoxicity

The expression of efflux-transporters in Calu-3 cell line was previously studied and confirmed [21-23]. To delineate the dose-dependent effect of PEITC on cytotoxic expressions of Calu-3 cells, IC50 values of PEITC alone and those in the presence of the efflux inhibitors (0.1 mM, 1 mM and 10 mM) were estimated (Fig. 5). There were significant differences in the cytotoxicity of PEITC loaded in either Calu-3 cells (~1.5 days), which expresses the efflux pump, or Human Embryonic Kidney cell line (~3.6 days), which is the absence of the efflux pump expression. The results of the cell cytotoxicity study revealed that cytotoxicity of PEITC loaded in CSM was greatly affected by the efflux pump activity and subsequently modulated by the efflux inhibitors. The estimated IC50 value of PEITC in the absence of the efflux inhibitors was 44.36 µM±2.61. An addition of 10 mM each of verapamil, nifedipine or tamoxifen, lowered the IC50 values of PEITC loaded in CSM to 25.92 µM, 22.65 µM and 14.64 µM, respectively. Cell cytotoxicity exerted by CSM seems to be due to the presence of the efflux inhibitors which enhanced PEITC accumulation through modulation of efflux-transporters.

Verapamil HCl and nifedipine (1 mM and 10 mM) in CSM significantly increased the cytotoxicity of PEITC loaded in CSM (Fig. 6A, B). The number of days to kill 50% of cells was reduced to 2.8±0.01 days and 2.6±0.2 days with 1 mM or 10 mM of verapamil HCl, respectively. Similarly, nifedipine lowered the number of days to kill 50% of cells from 3.9±0.08 days to 2.7±0.04 and 1.5±0.01 days for 1 mM or 10 mM of nifedipine, respectively. The concentration dependence on the cytotoxicity induced by PEITC in the presence of the efflux inhibitors in CSM suggests that the efflux transporters are involved in modulation of the accumulative amount of PEITC. A lack of any changes in cytotoxicity caused by PEITC in the presence of the inhibitors of a low concentration (0.1 mM) indicated that the threshold concentration of the inhibitors is required for the regulation of the efflux transporter mediated process.

Calcium channel blockers, like verapamil HCl and nifedipine, were found to regulate MRP or P glycoprotein expression by down regulating mRNA and post-transcriptional factors [24,25]. This activity may be contributable to the enhancement of cytotoxicity of PEITC loaded in CSM by both direct and indirect modulation of multi-drug resistant proteins. On the other hand, tamoxifen even at a low concentration (~0.1 mM) significantly increased the cytotoxicity of PEITC loaded in CSM (Fig. 6C). The number of days to kill 50% of cells was reduced from 3.8±0.08 days to 1.7±0.01, 1.1±0.08 and 0.8±0.01 days with 0.1 mM, 1 mM, 10 mM of tamoxifen, respectively (Fig. 6). An increase in cytotoxicity caused by loaded PEITC in the presence of tamoxifen may be due to self-induced cytotoxicity of tamoxifen. Since tamoxifen is known to cause DNA damage and PEITC terminates the phase 1 of ontogenesis, tamoxifen and PEITC may have synergistic effects on cellular cytotoxicity, which may need further studies.

5. Discussion

Chemotherapy encounters various barriers and frequently fails to achieve therapeutic concentrations in tumor cells [26-34]. The majority of the promising drugs currently available or in pipeline for the treatment of cancer are ineffective due to various physiological factors. The efflux transporters have recently received an increasing attention due to their broad spectrum of substrates and over expression in tumor cells. PEITC has demonstrated its effectiveness in metastatic breast cancer and small cell lung cancer [35,36]. Although PEITC is a potent anti-cancer agent, its usage is limited due to the low water solubility and high susceptibility to efflux-transporters in tumor cells. The protection of anti-cancer agents against efflux-transporters seems to be an ideal strategy for enhancement of their in vivo efficacies. A chronological or sequential delivery of efflux inhibitors and anti-cancer agents to the target site can be an alternative strategy to overcome efflux mediated restriction. Since PEITC inducing cell cycle arrest and apoptosis activities are time dependant processes [37], a prolonged exposure of PEITC to Calu-3 exerts more significant effects than multiple PEITC doses in chemotherapy. Therefore, this study was aimed to develop the formulation for PEITC that could be administered via pulmonary route for a prolonged period and enhance the therapeutic efficacy in the treatment of lung cancer.

Confocal microscopy revealed a uniform distribution of SLN in the matrix of CSM. The SLN were observed as blue dye interspersed within sulforhodamine stained chitosan with red fluorescence. The encapsulation efficiency in CSM for PEITC was 76% which was much higher than other highly lipophilic drugs like paclitaxel [38,39] or 5-fluoro uracil [40]. This higher encapsulation rate is mainly due to higher affinity of PEITC towards stearic acid [41]. The higher entrapment efficiencies of nifedipine, verapamil HCl and tamoxifen were also achieved with CSM as compared with other formulations [42-44]. In these novel formulations, the drug has to diffuse into two contrasting matrices before it is released into the external medium. The gradient formulation properties minimize the loosely bound drug distribution at the surface of the delivery system and also lowered the solubility across the polymeric system. The encapsulated drugs, such as nifedipine, verapamil HCl and tamoxifen, had an initial burst release accounted by the drug entrapment at the surface of the microparticle, whereas the lack of an initial burst release of PEITC suggested that the SLN were not released into the medium. The release patterns and its fitting to the mathematical equations indicated that SLN act as a reservoir of lipophilic drugs, resulting in a slower release of lipophilic drugs loaded in a composite matrix.

The release rates of the inhibitors were corroborated by the conventional mathematical models, whereas the release profile of PEITC from the CSM was not well correlated with them. The discrepancies in the release profiles in Fig. 4 were mainly due to the differences in aqueous solubility of each compound. Previous studies showed that lipophilic drugs had a slower release rate than hydrophilic drugs [45], indicating that the aqueous solubility of drugs was a major factor in defining the release rates from microparticles.

The cytotoxicity assay for free PEITC showed an IC50 value of 44.36 µM in Calu-3 cells. A rapid decrease in cell viability in Calu-3 cells was observed in the presence of the efflux transporter inhibitors at concentrations as low as 10 mM. An increase in cytotoxicity of Calu-3 cells by PEITC in the presence of inhibitors is indicative of the involvement of multi-drug resistant proteins in the drug-uptake process. Similar observations were made with Calu-3 cells in the presence of the efflux inhibitors, such as rhodamine 123, verapamil HCl, steroids and beta ligands [23,46]. The profiles of cytotoxicity of PEITC loaded in the CSM revealed a slower cell death rate as compared with those of free PEITC. The lower degree of cytotoxicity is in a good agreement with the slower release rate of PEITC from CSM, further supporting that the rate of cell death was one of the integral parameters in defining the efficacy of the controlled release formulations.

In the present work, we used the concentrations of 20 and 50 µM of PEITC, at which MRP inhibitors, such as verapamil, significantly increased the accumulation amount of PEITC by Calu-3 cells. Doses were selected based on the results that the treatment of PEITC (20 µM) induced JNK-mediated cell apoptosis in the transfected cells [47]. Since Calu-3 cells, which we used in this manuscript, express the
lower levels of P-gp than other cells, such as both Caco-2 and A549 cells [22,23], the results of the study performed on Calu-3 cells may be different from or even contrast to those tested in MCF-7/ADR (P-gp overexpressing) cells [19] and Caco-2 cells along with MDA435/LCC6 cells [18]. It should be also noted that even in the same cancer cells, tumors typically consist of a large therapy sensitive compartment and a smaller compartment with profound intrinsic resistance [48]. Therefore, it was speculated that PEITC is a substrate more sensitive to MRP2 than P-gp and subsequently the cell uptake study may have different results depending on type of cells in which the expression levels of P-gp and MRP are different.

It was previously reported that microparticles (1 to 10 µm) have the lower rate of particle internalization (about 10%), but still were noticeably taken by cells [49]. With the inclusion of the efflux transporter inhibitors in CSM, cytotoxicity increases at a rate similar to that assessed with free PEITC. The rate of cell death was observed to be dependent on not only the release rate of the efflux inhibitors but also the loading concentration of the inhibitors. Nelfipide (0.1 mM) and verapamil HCl (0.1 mM) showed no significant effects on cell cytotoxicity caused by PEITC. The higher concentrations of nelfipide (1 and 10 mM) enhanced cytotoxicity of PEITC on Calu-3 cells. The concentration dependent cytotoxicity in the presence of non-efflux inhibitors revealed that the cytotoxicity of Calu-3 cells caused by CSM was mediated by the efflux transporters. The results of cytotoxic study are well correlated with the release profiles of the entrapped drugs. An accumulation of cytotoxic drugs in the lung carcinoma cell has been significantly affected by the efflux transporters [50,51], which is in a good agreement with the results of the present study. For CSM in the absence of inhibitors, the cell death was not detected for 3.9 days until PEITC concentration in the medium was sufficiently high (66% of the loading dose). The cell death occurred at lower loading concentrations of PEITC, suggesting that the loss of PEITC by the efflux proteins is reduced and the toxic concentrations of PEITC were accumulated in a shorter period of time.

6. Conclusions

The results of this study demonstrated that Chitosan–SLN Micro-particles loaded with PEITC, an anti-cancer drug, and efflux transporter inhibitors, calcium channel blockers, significantly enhanced chemotherapeutic efficacy of PEITC. CSM provides mechanical strength as well as compatibility to an acidic, volatile and pungent PEITC. The particle size of CSM is small enough to be delivered through pulmonary inhalation to the target site. Cytotoxic expressions of Calu-3 cells exerted by PEITC in the presence of the efflux inhibitors can be attributable to the enhanced accumulation of PEITC through the modulation of the efflux transporter. CSM can serve as an efficient delivery system for chemotherapeutic agents susceptible to the multidrug resistant proteins.

References


